

AD _____

GRANT NUMBER DAMD17-96-1-6209

TITLE: The RIZ Gene in Human Breast Cancer

PRINCIPAL INVESTIGATOR: Shi Huang, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, CA 92037

REPORT DATE: September 1998

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE September 1998	3. REPORT TYPE AND DATES COVERED Final (1 Sep 96 - 31 Aug 98)	
4. TITLE AND SUBTITLE The RIZ Gene in Human Breast Cancer			5. FUNDING NUMBERS DAMD17-96-1-6209	
6. AUTHOR(S) Shi Huang, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Burnham Institute La Jolla, CA 92037			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			19981229 097	
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) The retinoblastoma protein-interacting zinc finger gene <i>RIZ</i> maps to the distal short arm of human chromosome 1 (1p36), a region thought to harbor tumor suppressor genes for a variety of human cancers including breast cancer. The <i>RIZ</i> gene normally produces two protein products of different length, RIZ1 and RIZ2. RIZ2 is generated by an internal promoter and lacks an amino-terminal motif of RIZ1, the PR domain conserved in a sub-family of zinc finger genes which function as negative regulators of tumorigenesis. We have here studied whether the <i>RIZ</i> gene may play a role in human breast cancer. We found that expression of RIZ1 is commonly decreased or at undetectable levels in breast cancer tissues and cell lines. Decreased RIZ1 expression was also found in other tumor types including neuroblastoma and lung cancer. Remarkably, RIZ2 is normally expressed in all cases examined, suggesting that the abnormality observed for RIZ1 is specific. Forced RIZ1 expression in breast cancer cells caused cell cycle arrest in G2/M and/or programmed cell death. These observations suggest an exclusive negative selection for RIZ1 but not RIZ2 in breast cancer and a role for RIZ1 in tumor suppression.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 23	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

NA Where copyrighted material is quoted, permission has been obtained to use such material.

NA Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

NA Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

NA In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

NA
PI - Signature

9-25-98

Date

TABLE OF CONTENTS
FINAL REPORT FOR GRANT NUMBER
DAMD17-96-1-6209

FRONT COVER	Page 1
REPORT DOCUMENTATION PAGE	Page 2
FOREWORD	Page 3
TABLE OF CONTENTS	Page 4
INTRODUCTION	Pages 5
BODY	Pages 5-9
CONCLUSIONS	Pages 9-10
REFERENCES	Pages 10-12
APPENDICES	N/A

The RIZ gene in Human Breast Cancer, Final Report 1998, PI. Shi Huang

Introduction:

The retinoblastoma protein (Rb)-interacting zinc finger gene *RIZ* was isolated in a functional screening for Rb-binding proteins [1], and independently as a *GATA-3*-binding protein *G3B* [2] and as a DNA-binding protein *MTB-Zf* [3]. *RIZ* contains extensive sequence and antigenic similarity to the E1A viral oncoprotein, including the LXCXE Rb-binding motif [4]. The physiological role of the binding to Rb is not yet defined because relatively little is known about the biological function of *RIZ*. There are several other interesting motifs in *RIZ*, including eight zinc finger motifs and a PR (*PRDI-BF1-RIZ* homology) domain of ~100 amino acids which defines a sub-family of Kruppel-like zinc finger genes. The *RIZ* gene normally produces two different products, RIZ1 and RIZ2, which differ in length by the presence or absence of the PR domain [5]. An internal promoter generates RIZ2 which lacks the amino terminal PR domain of RIZ1 but is otherwise identical to RIZ1. Both products are widely expressed in normal tissues [5]. They are both located in the nucleus and may function as GC-element DNA binding transcription repressors [6], or as MTB-element binding activators [3]. Other members of the PR family include the *MDS1-EV11* breakpoint gene involved in human leukemia and the *PRDI-BF1* or *BLIMP1* transcription repressor which can drive B cell maturation [7-10]. *RIZ* is remarkably similar to *MDS1-EV11* which also gives rise to a PR lacking product, the EV11 oncoprotein, through an internal promoter [7]. PR domain appears to function as a protein binding interface and is related to the SET domain involved in chromatin-mediated gene expression [11].

Several lines of observation suggest that PR genes or the PR-containing products of these genes are negative regulators of cell growth and tumorigenesis. The PR region of *MDS1-EV11* is often disrupted by leukemia associated chromosomal insertions and translocations, resulting in the activation of the PR deficient *EV11* gene which is considered oncogenic [12]. *MDS1-EV11* is functionally different from EV11, which may be the basis for the selective activation of EV11 in tumor cells [13]. A tumor suppressor role for *RIZ* is suggested by its chromosomal location at the distal short arm of human chromosome 1 next to the marker D1S228 on 1p36.23 [3, 14], which commonly undergoes deletions, rearrangements, or loss of heterozygosity in a broad spectrum of human tumors, including those of breast [15, 16], liver [17], colon [18], and neurocrest tissues [19, 20].

To assess the role of *RIZ* gene in tumorigenesis, we have examined *RIZ* gene expression in tumor tissues and tissue derived cell lines. The results show that RIZ1 expression is decreased or lost in human breast cancer whereas RIZ2 expression is normal. Consistent with what appears to be negative selection for RIZ1 in tumor cells, forced expression of RIZ1 causes cell cycle arrest in G2/M and/or apoptosis.

Materials and Methods:

RNase protection, RT-PCR, immunoblot, and *in situ* hybridization analysis.

Human cell lines were grown in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal calf serum. RNA was prepared and used for RNase protection analysis as described previously [5]. For RT-PCR analysis, reverse transcription was performed using M-MLV reverse transcriptase (NEB) and oligonucleotide primer RP217 located in coding exon 7 (5'-CCT CTG AGC AGT CTT CAA GAG T-3'). The first strand cDNA sample was then amplified using two different sets of primers, one set (RP168 + RP217) was specific for RIZ1 and the other (RP216 + RP217) for RIZ2+RIZ1. RP168 primer is located within exon 4 (5'-TGG CTG CGA TAT GTG AAT TG-3'). RP216 primer is located within exon 6 (5'-CAA CTG AAG ACA AGT GAG CCA GA-3'). The primers for amplification of human beta-actin are 5'-GTGGGGCGCCCCAGGCACCA-3' and 5'-CTCCTTAATGTCACGCACGATTTC-3'. PCR reactions were run for 15-20 cycles. The PCR products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining or by Southern blotting using RIZ cDNA probe. Band intensities were quantitated using the Molecular Analysis software and a Phospho-

imager (Bio-Rad). Immunoprecipitation and immunoblot analysis of RIZ proteins were performed as described previously [1, 5]. *In situ* hybridization analysis of fixed breast tissues was carried out as described previously using digoxigenin-labeled probes [21]. The RIZ1 antisense probe was a 0.6 kb fragment generated by SP6 transcription of the amino terminal region of RIZ1 upstream of coding exon 1 [5]. The exon 7 antisense probe was a 0.5 kb fragment corresponding to amino acids 700-866 and was generated by Sp6 transcription.

Construction of recombinant adenovirus vector encoding RIZ1.

The full length rat RIZ1 cDNA in the plasmid p3RIZr was isolated by Nru1 and Xho1 digestion and was cloned into the vector pXCJL1[22]. The resulting plasmid pAdRIZ1 and the adenovirus packaging plasmid pJM17 [22] were cotransfected into the E1 trans-complementing cell line 293 using calcium phosphate. High-titered stocks of recombinant adenoviruses were grown in 293 cells and purified by density gradient ultracentrifugation. Adenovirus mediated RIZ1 expression was examined by immunoblot analysis.

Development of cell lines with inducible RIZ1 expression

The cell line UTA6 derived from U2OS was a kind gift of Dr. D. Haber [23]. Construct ptetRIZ1 was constructed by cloning the full-length rat RIZ1 cDNA into the vector pUHD10-3 kindly provided by Dr. H. Bujard [24]. The founder cell lines were transfected with ptetRIZ1 and pBK-hygro. Drug resistant colonies were isolated in the presence of tetracycline, and screened for RIZ1 expression by immunostaining or immunoblot upon withdrawal of tetracycline. Three clones demonstrating regulated induction of the transfected gene were selected for further study, and representative results were described for one of these, RIZ1-29.

Growth, cell cycle, and apoptosis assays

Cells were seeded at 2×10^5 cells in 6 cm dishes. After overnight culture, the cells were incubated with recombinant adenovirus for 1 hr at a MOI of 50 in serum free media. The virus containing media was then supplemented with serum to 10%, and the culture was continued for various times up to 72 hr. At each time point, live cell numbers were counted by trypan blue exclusion, and all cells were processed for DNA histogram analysis as follows. Cells were detached from the plate by trypsin and were pooled with cells in the culture medium. These cells were washed twice with PBS, and were fixed with 70% ethanol overnight. Cells were then washed once in PBS and incubated in propidium iodide solution (50 μ g/ml in PBS) containing RNase (10 μ g/ml) for at least 30 min. The stained cells were analyzed with a FACscan (Becton-Dickinson) using a CellQuest program.

DNA fragmentation was performed 72 hr after infection with recombinant adenovirus. Adherent and nonadherent cells were pooled and genomic DNA was isolated and analyzed using standard procedures. TUNEL analysis was performed as described [25]. Cells grown on coverslips were infected with adenovirus and were fixed 72 hr post infection. Apoptotic cells were labeled with biotin-conjugated dUTP using terminal deoxynucleotidyl transferase (Boehringer Mannheim) and fluorescein-conjugated ExtrAvidin (Sigma), and visualized by fluorescent confocal microscopy. To demonstrate nuclear fragmentation, cells were co-stained with propidium iodide (250 μ g/ml) for 5 min at room temperature before the final washes.

Results:

Decreased RIZ1 expression in breast cancer

To study whether the *RIZ* gene may play a role in human neoplasia, we first examined the expression of the two alternative forms of *RIZ*, RIZ1 and RIZ2, in breast cancer cell lines using a previously described RNase protection assay [5]. A representative result of this analysis is shown in Fig. 1A. RIZ1 and RIZ2 mRNAs were found in an immortalized normal mammary epithelial cell line 184A1N4. However, two breast cancer cell lines, SK-BR-3 and MDA-MB-435, had

reduced or undetectable levels of RIZ1 relative to beta-actin. In contrast, RIZ2 transcripts were readily detected and no significant differences in RIZ2 levels were observed. This underexpression of RIZ1 but not RIZ2 was also confirmed by RT-PCR analysis as in Fig. 1B, which showed loss of RIZ1 in MDA-MB-435 and decreased RIZ1 expression in SK-BR-3 and MDA-MB-231 cells. Immunoblot analysis showed that RIZ2 protein was normal consistent with mRNA expression (Fig. 1C). RIZ1 protein was normally expressed at lower levels than RIZ2 as seen in 184A1N4 cells. Reduced RIZ1 protein levels consistent with lowered mRNA levels was observed in SK-BR-3 cells. We examined a large set of cell lines or tissues of various tumor types by RNase protection and RT-PCR assays. Similar levels of RIZ2 mRNA were found in all cases examined. In contrast, low or absent RIZ1 mRNA expression was found in 4 of 8 breast cancer cell lines, 2 of 5 lung cancer cell lines, 4 of 14 neuroblastoma cell lines and tissue samples, and 1 of 2 osteosarcoma cell lines (Table 1). The results indicate that decreased expression of RIZ1 but not RIZ2 may be a common feature for a subset of human cancers.

To further confirm loss of RIZ1 mRNA expression in tumor tissues, we performed *in situ* analysis of RIZ gene expression in breast carcinoma specimens. Fixed breast sections from 5 infiltrating carcinomas and 7 benign breast lesions (reduction mammoplasty specimens) were examined using procedures described previously [21]. Anti-sense probe derived from a 5'-end cDNA fragment of RIZ1 was used to specifically hybridize to RIZ1 mRNA. Because RIZ2 mRNA does not contain unique sequences not present in RIZ1, a cDNA fragment of coding exon 7 was used to detect both RIZ1 and RIZ2 transcripts. Positive RIZ1 hybridization signal was found in normal epithelial cells in 6 of 7 normal breast tissues or benign lesions. No RIZ1 expression was found in the neoplastic epithelial cells in 4 of 5 infiltrating breast carcinomas. Representative positive RIZ1 hybridization in normal ductal breast epithelial cells and in normal lobular cells are shown in Fig. 2A-C (2C but not 2A-B were counter stained with hematoxylin). Representative negative RIZ1 hybridization in carcinoma samples is shown in Fig. 2D (with hematoxylin counter-stain). Similar sections of the same normal (Fig. 2E) or tumor samples (Fig. 2F) showed positive staining for the coding exon 7 probe, indicating RIZ2 expression in transformed breast epithelial cells. We conclude from these studies that RIZ1 underexpression may be a common change associated with breast cancer.

Forced RIZ1 expression induces cell growth arrest and/or apoptosis

The specific underexpression of RIZ1 in tumors suggests that RIZ1 function may be incompatible with tumor cell growth. To explore this possibility, we developed a recombinant adenovirus, AdRIZ1, that lacks the E1A and E1B regions and contains a rat RIZ1 cDNA transcribed from the cytomegalovirus (CMV) promoter. An osteosarcoma cell line U2OS which expressed low levels of RIZ1 was initially used for determining the effects of RIZ1 expression. Adenovirus-mediated RIZ1 protein expression can be detected at 24 hr and peaked at 48 hr post virus infection (Fig. 3A). AdRIZ1 infected cells grew more slowly than cells infected with control virus AdGFP (expressing green fluorescent protein) and cell death became apparent 72 hr post AdRIZ1 infection (Fig. 3B).

To examine RIZ1 activity on cell cycle progression, DNA histogram analysis was performed on AdRIZ1 infected U2OS cells (Fig. 3C and D). The results showed that a majority of cells were arrested at G2/M at 48 hr post AdRIZ1 virus infection. At 72 hr post infection, a significant fraction of AdRIZ1 infected cells had sub-G1 DNA content indicating apoptotic cell death. In contrast, AdGFP virus infected cells were mostly at G1 and did not show sub-G1 DNA content. In addition to the sub-G1 DNA content, several other observations also show that RIZ1 induced cell death through the apoptotic process. DNA from dying cells 72 hr post infection revealed fragmentation of chromatin into nucleosome ladders (Fig. 3E). Double staining of individual cell nuclei with propidium iodide and TUNEL (DNA terminal transferase nick-end translation) reagents demonstrated nuclear condensation and fragmentation, and presence of free DNA 3'-OH ends (Fig. 3F) [25]. Together, these results suggest that RIZ1 inhibits cell proliferation by inducing cell cycle arrest in G2/M and programmed cell death.

U2OS osteosarcoma cells express wild type Rb and p53 proteins. We next examined whether RIZ1 can also induce growth arrest and cell death in SAOS2 osteosarcoma cells that do

not express wild type Rb and p53 proteins. Similar to results in Fig. 3, AdRIZ1 virus infection of SAOS2 cells also led to accumulation of cells with G2/M DNA content and with sub-G1 DNA content (Table 2). The results show that RIZ1 can induce G2/M arrest and apoptosis in the absence of p53 and Rb proteins.

We also expressed RIZ1 in breast cancer cell lines as shown in Table 2. AdRIZ1 infection led to cell death but no significant G2/M arrest in SK-BR-3 cells. In contrast, primarily G2/M arrest but no cell death was found for T47D cells infected with the same amount of AdRIZ1 virus. The results show that forced RIZ1 expression induced cell death or G2/M arrest in breast cancer cells.

To study the role of RIZ1 in cell growth control using a different approach, we established RIZ1-inducible cell lines, using a tetracycline-regulated transactivator [24]. In this system, expression of a tetracycline-repressible transactivator allows tight regulation of a promoter containing tet operator sequences. Construct containing RIZ1 under control of this promoter was stably transfected into founder cell lines expressing the transactivator. The U2OS derived founder cell line UTA6 was used to derive RIZ1 expressing cell lines [23]. Results from a representative cell line tetRIZ1.29 are presented in Fig. 4. Withdrawal of tetracycline led to induction of RIZ1 expression, detectable at 9 hr and peaking at 24 hr. While uninduced cells grew normally, RIZ1 expressing cells grew slower and by 4 days post induction of RIZ1, only 5% of the cells survived (Fig. 4). DNA histogram analysis showed G2/M delay and apoptosis (data not shown). These results are consistent with those described for adenovirus mediated RIZ1 expression.

Discussion:

A role for RIZ1 but not RIZ2 product of the *RIZ* locus as a negative regulator of tumorigenesis is suggested by two previous observations: *RIZ* maps to human chromosome band 1p36 and the RIZ1-related MDS1-EVI1 gene is often disrupted in myeloid leukemia. We provide here several direct lines of observation further supporting a role for RIZ1 as a suppressor of cell growth and tumorigenesis.

Our data indicate that underexpression of RIZ1 but not RIZ2 may be common in human breast cancer. RIZ1 underexpression was also found in neuroblastoma and lung cancer, and may be expected to occur in other malignancies as well. In contrast to RIZ1, the alternative product of the same gene, RIZ2, is uniformly present in all normal and tumor samples examined. We are aware that it is not uncommon for tumor cells to show non-specific changes in the expression of a particular gene. However, the exclusive alteration of one but not the other product of the same gene is highly unusual. The uniform presence of RIZ2 suggest that loss of RIZ1 is not a random-occurring event. There may be a specific negative selection for RIZ1 versus RIZ2 in tumors. Since the PR domain is similar to the SET domain involved in chromatin remodeling [11], it is likely that RIZ1 and RIZ2 may differ significantly in their functions in regulating chromatin-mediated gene expression. This may underlie the apparently opposite roles of RIZ1 and RIZ2 in tumorigenesis.

Inactivating gene expression rather than mutating protein structure appears to be the basis of RIZ1 alteration in malignant cells. Southern blot analysis did not show evidence of gross alterations of RIZ1 genomic DNA in tumor cells (not shown). Whether tumor associated 1p36 alterations may inactivate RIZ1 expression requires future investigation. Relative to the RIZ1 abnormality, the uniform presence of RIZ2 is striking, and may indicate a need for RIZ2 in oncogenesis. Two alternative forms of the RIZ1-related PR domain gene MDS1-EVI1 have previously been proposed to play opposite functional roles in transformation [5, 13]. A need to maintain RIZ2 expression in tumor cells may explain the lack of gross mutations in *RIZ* because RIZ2 shares 89% of coding region with RIZ1. Of course, mutations in the PR region of RIZ1 should not affect RIZ2. Such mutations, however, must be subtle (undetectable by Southern-blot analysis). Moreover, if such mutations exist, they are likely to be rare because tumors primarily display RIZ1 underexpression. However, it is possible that certain nucleotide changes could lead to destabilization of transcripts. Given that RIZ1 and RIZ2 are produced by different promoters, it seems likely that RIZ1 promoter may represent a specific target of inactivation in tumor cells. Future study of this promoter may reveal insights into the decreased RIZ1 expression in tumors.

The activity of RIZ1 in inducing cell cycle arrest and/or apoptosis described here provides a molecular mechanism for a negative role of RIZ1 in tumorigenesis. RIZ1 induced both cell cycle arrest and cell death in certain cell lines (U2OS and SAOS2) but either arrest or apoptosis in other cell lines (arrest in T47D and apoptosis in SK-BR-3). The data suggest that induction of apoptosis by RIZ1 is unlikely a result of induction of cell cycle arrest. Also, RIZ1 induced cell death independent of the endogenous expression of Rb and p53, suggesting that RIZ1 can function independent of Rb and p53. However, the data do not exclude the possibility that RIZ1 function may be regulated by Rb and p53. The amount of RIZ1 expressed by adenovirus may exceed that of endogenous Rb or p53 for their effects on RIZ1 to be observed. Forced RIZ1 expression also induced apoptosis and growth arrest independent of endogenous levels of RIZ1. This is not unexpected because forced expression of Rb or p53 has also been shown to cause cell growth inhibition independent of endogenous levels of expression [26, 27].

That RIZ1, which binds to Rb, induces apoptosis suggests a potential mechanism of RIZ1 action in the context of the Rb suppressor pathway. Several lines of investigation show that Rb plays a role in inhibiting apoptosis [28-30]. The apoptosis inducing activity of E2F1, which is negatively regulated by Rb [30], is thought to mediate E2F1's role as a tumor suppressor [31]. Clearly, it is an important future goal to determine whether Rb may inhibit RIZ1-mediated apoptosis.

Recommendations in relation to the Statement of Work:

Our revised Statement of Work has two technical objectives. We believe that we have now largely accomplished these objectives in a positive fashion. Our results suggest that the hypothesis that this proposal is addressing is likely to be correct.

Objective 1 studies whether RIZ gene alteration occurs in breast cancer. We have uncovered one form of RIZ gene alteration to be common in breast cancer. Thus, one of the two products of RIZ gene, RIZ1, is underexpressed in breast cancer. Given the limited resources, we have only performed a small scale screening of RIZ1 gene mutation screen which have not yet uncovered any mutations in RIZ gene except some apparent polymorphisms. Also, given our focus on the loss of RIZ1 expression analysis, the experiments on allelic expression were beyond our capacity to do. We believe that our most important objective has been accomplished which is to establish an alteration of RIZ in breast cancer.

Objective 2 (revised) studies tumor suppression by RIZ1 in cell culture and in nude mice. We have constructed a recombinant adenovirus expression RIZ1. This virus was shown to cause growth arrest and apoptosis in breast cancer cells in tissue culture. Experiments using nude mice tumor models are in progress.

Conclusions:

Our results suggest that alteration of RIZ1 may play an important role in breast cancer development. RIZ1 is commonly underexpressed in breast cancer, and forced RIZ1 expression can induce breast cancer cell cycle arrest and apoptosis. The data identify a new candidate breast cancer suppressor. Given that RIZ1 gene lacks apparent mutations in cancer, we are establishing knock-out animal models in order to prove unequivocally that loss of RIZ1 causes tumorigenesis. Based on the encouraging results so far from the RIZ1-deficient animal model, we believe that RIZ1 is an authentic tumor suppressor in both human and mice. Recent data also indicate a role for RIZ1 in connecting the Rb and p53 tumor suppressor pathways, which would explain the anti-neoplastic function of RIZ1. Future study of RIZ1 would advance our understanding of the Rb and p53 pathway. RIZ1 has real implications for breast cancer prevention and treatment. The recombinant RIZ1 adenovirus can be directly used for clinical trials of breast cancer gene therapy. We have demonstrated the anti-growth power of this virus in tissue cultured breast cancer cells. We are presently testing the tumor suppression activity of this virus in nude mice tumor models.

Papers from this grant:

1. He, L., Yu, J. X., Liu, L., Buyse, I.M., Wang, M.-S., Yang, Q., Nakagawara, A., Brodeur, G.M., Shi, Y.E., and Huang, S. (1998) RIZ1, but not the alternative RIZ2 product of the same gene, is underexpressed in breast cancer and forced RIZ1 expression causes G2/M cell cycle arrest and/or apoptosis. *Cancer Research*, in press (Oct 1, 1998 issue).

Abstracts:

1. He, L., Yu, J. X., Liu, L., Buyse, I.M., Wang, M.-S., Yang, Q., Nakagawara, A., Brodeur, G.M., Shi, Y.E., and Huang, S. Induction of p53 and apoptosis by the candidate tumor suppressor RIZ1. DOD BCRP meeting Era of Hope, Oct 31-Nov. 4, 1997. Washington, DC
2. He, L., Yu, J. X., Liu, L., Buyse, I.M., Wang, M.-S., Yang, Q., Nakagawara, A., Brodeur, G.M., Shi, Y.E., and Huang, S. Induction of p53 and apoptosis by the candidate tumor suppressor RIZ1. (oral presentation), Cancer genetics and tumor suppressor gene Conference, June 12-16, Frederick, Maryland.

Personnel:

Shi Huang, Ph.D., Principal Investigator
Jack Yu, Ph.D., Postdoctoral Associate
Liusheng He, M.D., Ph.D., Postdoctoral Associate

References:

1. Buyse, I. M., Shao, G., and Huang, S. The retinoblastoma protein binds to RIZ, a zinc finger protein that shares an epitope with the adenovirus E1A protein. *Proc. Natl. Acad. Sci. USA*. 1995;92: 4467-4471.
2. Shapiro, V. S., Lee, P., and Winoto, A. Identification and cloning of the G3B cDNA encoding a 3' segment of a protein binding to GATA-3. *Gene* 1995;163: 329-330.
3. Muraosa, Y., Takahashi, K., Yoshizawa, M., and Shibahara, S. cDNA cloning of a novel protein containing two zinc-finger domains that may function as a transcription factor for the human heme-oxygenase-1 gene. *Eur. J. Biochem.* 1996;235: 471-479.
4. Buyse, I. M. and Huang, S. In Vitro Analysis of the E1A-Homologous Sequences of RIZ. *J. Virol.* 1997;71: 6200-6203.
5. Liu, L., Shao, G., Steele-Perkins, G., and Huang, S. The retinoblastoma interacting zinc finger gene RIZ produces a PR domain lacking product through an internal promoter. *J. Biol. Chem.* 1997;272: 2984-2991.
6. Xie, M., Shao, G., Buyse, I.M., Huang, S. Transcriptional repression mediated by the PR domain zinc finger gene RIZ. *J. Biol. Chem.* 1997;272: 26360-26366.
7. Fears, S., Mathieu, C., Zeleznik-Le, N., Huang, S., Rowley, J. D., and Nucifora, G. Intergenic splicing of *MDS1* and *EVII* occurs in normal tissues as well as in myeloid leukemia and produces a new member of the PR domain family. *Proc. Natl. Acad. Sci. USA*. 1996;93: 1642-1647.
8. Keller, A. D. and Maniatis, T. Identification and characterization of a novel repressor of beta-interferon gene expression. *Genes Dev.* 1991;5: 868-879.
9. Turner, C. A., Jr., Mack, D. H., and Davis, M. M. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. *Cell* 1994;77: 297-306.
10. Huang, S. Blimp-1 is the murine homolog of the human transcriptional repressor PRDI-BF1 [letter]. *Cell* 1994;78: 9.

11. Huang, S., Shao, G., and Liu, L. The PR Domain of the Rb-binding Zinc Finger Protein RIZ1 is a Protein Binding Interface and Is Related to the SET Domain Functioning in Chromatin-mediated Gene Expression. *J. Biol. Chem.* 1998;273.: 15933-15940.
12. Morishita, K., Parker, D. S., Mucenski, M. L., Jenkins, N. A., Copeland, N. G., and Ihle, J. N. Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. *Cell* 1988;54: 831-840.
13. Soderholm, J., Kobayashi, H., Mathieu, C., Rowley, J. D., and Nucifora, G. The leukemia-associated gene MDS1/EVI1 is a new type of GATA-binding transactivator. *Leukemia* 1997;11: 352-8.
14. Buyse, I. M., Takahashi, E., and Huang, S. Physical mapping of the retinoblastoma-interacting zinc finger gene RIZ to D1S228 on chromosome 1p36. *Genomics* 1996;34: 119-121.
15. Nagai, H., Negrini, M., Carter, S. L., Gillum, D. R., Rosenberg, A. L., Schwartz, G. F., and Croce, C. M. Detection and cloning of a common region of loss of heterozygosity at chromosome 1p in breast cancer. *Cancer Res.* 1995;55: 1752-1757.
16. Genuardi, M., Tsihira, H., Anderson, D. E., and Saunders, G. F. Distal deletion of chromosome 1p in ductal carcinoma of the breast. *Am.J.Hum.Genet.* 1989;45: 73-82.
17. Simon, D., Knowles, B. B., and Weith, A. Abnormalities of chromosome 1 and loss of heterozygosity on 1p in primary hepatomas. *Oncogene.* 1991;6: 765-770.
18. Bardi, G., Pandis, N., Fenger, C., Kronborg, O., Bomme, L., and Heim, S. Deletion of 1p36 as a primary chromosomal aberration in intestinal tumorigenesis. *Cancer Res.* 1993;53: 1895-1898.
19. Dracopoli, N. C., Harnett, P., Bale, S. J., Stanger, B. Z., Tucker, M. A., Housman, D. E., and Kefford, R. F. Loss of alleles from the distal short arm of chromosome 1 occurs late in melanoma tumor progression. *Proc. Natl. Acad. Sci. USA.* 1989;86: 4614-4618.
20. Fong, C. T., White, P. S., Peterson, K., Sapienza, C., Cavenee, W. K., Kern, S. E., Vogelstein, B., Cantor, A. B., Look, A. T., and Brodeur, G. M. Loss of heterozygosity for chromosomes 1 or 14 defines subsets of advanced neuroblastomas. *Cancer Res.* 1992;52: 1780-1785.
21. Ji, H., Liu, Y. E., Jia, T., Wang, M., Liu, J., Xiao, G., Joseph, B. K., Rosen, C., and Shi, Y. E. Identification of a breast cancer-specific gene, BCSG1, by direct differential cDNA sequencing. *Cancer Res.* 1997;57: 759-64.
22. McGrory, W. J., Bautista, D. S., and Graham, F. L. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology* 1988;163: 614-7.
23. Englert, C., Hou, X., Maheswaran, S., Bennett, P., Ngwu, C., Re, G. G., Garvin, A. J., Rosner, M. R., and Haber, D. A. WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis. *EMBO J.* 1995;14: 4662-4675.
24. Gossen, M. and Bujard, H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA.* 1992;89: 5547-5551.
25. Ben-Sasson, S. A., Sherman, Y., and Gavrieli, Y. Identification of dying cells--in situ staining. *Methods Cell Biol* 1995;46: 29-39.
26. Fung, Y. K., T'Ang, A., Murphree, A. L., Zhang, F. H., Qiu, W. R., Wang, S. W., Shi, X. H., Lee, L., Driscoll, B., and Wu, K. J. The Rb gene suppresses the growth of normal cells. *Oncogene.* 1993;8: 2659-2672.
27. Gomez-Manzano, C., Fueyo, J., Kyritsis, A. P., Steck, P. A., Roth, J. A., McDonnell, T. J., Steck, K. D., Levin, V. A., and Yung, W. K. Adenovirus-mediated transfer of the p53 gene produces rapid and generalized death of human glioma cells via apoptosis. *Cancer Res.* 1996;56: 694-9.
28. Macleod, K. F., Hu, Y. W., and Jacks, T. Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. *EMBO J.* 1996;15: 6178-6188.

29. Haas-Kogan, D. A., Kogan, S. C., Levi, D., Dazin, P., T'Ang, A., Fung, Y. K., and Israel, M. A. Inhibition of apoptosis by the retinoblastoma gene product. *EMBO J.* 1995;14: 461-472.
30. Hsieh, J.-K., Fredersdorf, S., Kouzarides, T., Martin, K., and Lu, X. E2F1-induced apoptosis requires DNA binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction. *Genes Dev.* 1997;11: 1840-1852.
31. Field, S. J., Tsai, F. Y., Kuo, F., Zubiaga, A. M., Kaelin, W. G., Jr., Livingston, D. M., Orkin, S. H., and Greenberg, M. E. E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* 1996;85: 549-561.

Appendices:**Table 1. Analysis of *RIZ* gene expression.**

^a RT-PCR and RNase protection assay (RPA) as described in Fig. 1 were performed on total RNAs from cell lines and tissues as listed. *RIZ2* transcripts can not be differentiated from *RIZ1* by RT-PCR assay; *RIZ2*+*RIZ1* represents *RIZ* gene common exon 7 derived transcripts. To compare *RIZ1* and *RIZ2* levels of different samples, beta-actin mRNA expression was used as internal control for variations in the amount of RNA used. The ratio of *RIZ1* to beta-actin of each tumor sample was quantitated and was normalized to that of 184A1N4 cells which was arbitrarily defined as 1. The normalized ratio is shown. Similarly, the ratio of *RIZ2* or *RIZ2*+*RIZ1* versus beta-actin of each sample was normalized to that of 184A1N4 cells and the normalized ratio is shown. ND, not done.

^b Samples with the *MYCN* gene amplification.

Table 2. Forced *RIZ1* expression in osteosarcoma and breast cancer cell lines.

^a 72 hours post Ad*RIZ1* or AdGFP virus infection, DNA histogram analysis was performed as described in Fig. 3. Value shown represents difference in percentage of cell populations between Ad*RIZ1* and AdGFP infected cells. Increase caused by Ad*RIZ1* over AdGFP is indicated by a plus sign, and decrease by a minus sign. Value represents mean with standard deviation of three independent experiments.

Figure Legends:

Fig. 1. Analysis of RIZ gene expression. (A) RNase protection analysis of normal breast epithelial cell line (184A1N4) and breast cancer cell lines (SK-BR-3, MDA-MB-435, and BT474) as indicated on top of each lane. (B) RT-PCR analysis of RIZ gene expression in breast cancer cell lines. RIZ2 transcripts can not be differentiated from RIZ1 by RT-PCR assay because of their sequence overlap; RIZ1+RIZ2 represents PCR products derived from the overlapping common coding-exon 7. Shown are Southern blots of RIZ gene PCR products and ethidium bromide stained PCR products of beta-actin. (C) Immunoblot analysis of RIZ protein expression in breast cancer cell lines as indicated. Cell extracts were prepared from exponentially growing cells and were immunoprecipitated with rabbit serum recognizing both RIZ1 and RIZ2 proteins. The precipitated proteins were analyzed on 5% SDS gel followed by immunoblot using mouse serum recognizing both RIZ1 and RIZ2 proteins.

Fig. 2. RIZ gene expression in human breast tissues by *in situ* hybridization. RIZ1 probe hybridized to normal ductal breast epithelial cells (A) and normal lobular cells (B), shown without hematoxylin counter stain. RIZ1 probe hybridized to normal ductal breast epithelial cells (C) but not to breast carcinoma cells (D), shown with hematoxylin counter stain. Exon 7 probe hybridized to normal ductal breast epithelial cells (E) and to carcinoma cells (F), shown without hematoxylin counter stain. Arrows indicate cells showing positive hybridization signals.

Fig. 3. Induction of cell growth arrest and cell death by RIZ1. (A) RIZ1 protein expression in AdRIZ1 infected cells. U2OS cells infected with AdRIZ1 or AdGFP virus (50 MOI) and cell lysates were analyzed by 5% SDS gel analysis followed by immunoblot using RIZ1 antibodies. (B) Growth rate analysis of AdRIZ1 infected U2OS cells. Live cell numbers were counted at different days post virus infection. Shown is a representative of three independent experiments. (C) DNA histogram analysis. At different times post adenovirus infection, cells were analyzed for DNA content by propidium iodide staining followed by FACScan. A representative of three independent experiments is shown. (D) Quantitative analysis of the number of cells in different phases of the cell cycle for the experiment shown in (C). A: Sub-G1 or apoptosis peak. (E) DNA fragmentation gel electrophoresis analysis was performed at 72 hr post virus infection of U2OS cells. (F) TUNEL analysis of AdRIZ1 infected cells. 72 hr post virus infection, cells were fixed and the fixed cells were double-stained with propidium iodide (PI) and FITC-labeled TUNEL reagents as described in Methods. Shown are images from a fluorescent confocal microscope.

Fig. 4. Growth inhibition by inducible RIZ1 expression. (A) RIZ1 protein expression in RIZ1-29 cells induced by withdrawal of tetracycline (tet). Protein extracts were prepared at different times following tetracycline withdrawal and were analyzed by immunoblot analysis. (B) Growth curve of RIZ1-29 cells in the presence or absence of tetracycline. Live cells were counted at each time point using the trypan blue exclusion method.

Table 1. Analysis of RIZ gene expression

Samples	Tissue types	RT-PCR	RT-PCR	RPA	RPA
		RIZ1/actin ^a	(RIZ2+RIZ1)/actin	RIZ1/actin	RIZ2/actin
Normal tissues and cell lines					
184A1N4	mammary cell line	1	1	1	1
F20	placental tissue	1.12	1.21	1.11	1.16
WI38	lung fibroblast	0.87	0.82	0.91	0.85
Breast cancer cell lines					
MB231		0.11	1.02	0.1	1.05
MB468		0.14	1.13	0.12	1.08
MCF7		0.87	0.97	0.92	0.97
T47D		0.94	0.93	0.96	0.95
SK-BR-3		0.07	1.21	0.18	1.32
MB435		0	1.22	0	1.24
BT474		ND	ND	1.1	0.98
BT549		ND	ND	0.92	1
Neuroblastoma cell lines					
LAN-1(MYCN) ^b		0.96	0.95	1.02	0.94
SHEP-17		1.03	1.02	0.98	1.21
SK-N-BE2 (MYCN)		0.98	1.12	0.93	0.97
IMR-32 (MYCN)		1.21	1.01	1.15	1.07
SY5Y		0.17	1.02	ND	ND
NGP (MYCN)		0.97	1.03	1	0.98
SMS-KCN (MYCN)		0.12	1.14	ND	ND
Primary neuroblastoma					
N-31	stage I	1.02	1.1	ND	ND
N-59		0.96	1.02	ND	ND
N-106	stage II	0.15	1.01	ND	ND
N-63	stage III	1.02	0.98	ND	ND
N-111		1.1	1.02	ND	ND
N-11	stage IV	0.99	1.14	ND	ND
N-75 (MYCN)	stage IVS	0.11	0.96	ND	ND
Lung cancer cell lines					
A549		1.01	0.97	ND	ND
Calu6		1.1	1.08	ND	ND
H460		0.98	0.99	ND	ND
H292		0.1	1.21	ND	ND
H441		0	1.02	0	1.13
Osteosarcoma cell lines					
U2OS		0.12	0.96	ND	ND
SAOS2		1.14	1.09	ND	ND

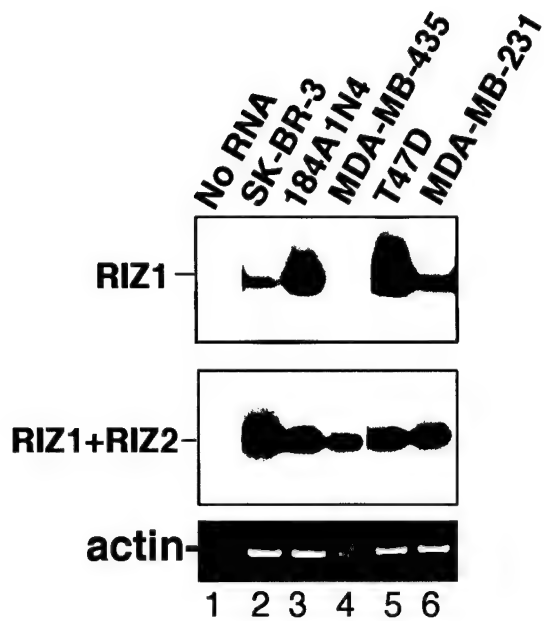
Table 2. Forced RIZ1 expression in osteosarcoma and breast cancer cell lines

Cell Lines	Apoptosis	G1	S	G2/M	Rb	p53	RIZ1
U2OS	+17.4 ± 3.1 ^a	-29.7 ± 4.6	-7.0 ± 2.1	+20.1 ± 3.0	+	+	weak
SAOS2	+20.3 ± 4.2	-36.1 ± 5.2	-1.1 ± 2.0	+14.9 ± 3.7	-	-	+
SK-BR-3	+41.5 ± 7.7	-33.8 ± 4.0	-5.2 ± 3.3	+1.2 ± 0.8	+	mut	weak
T47D	+1.2 ± 1.0	-44.0 ± 8.3	-1 ± 0.6	+43 ± 7.8	+	mut	+

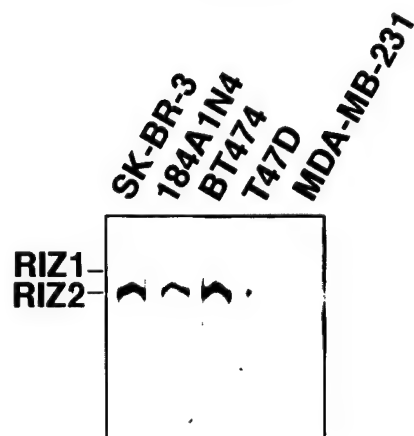
A

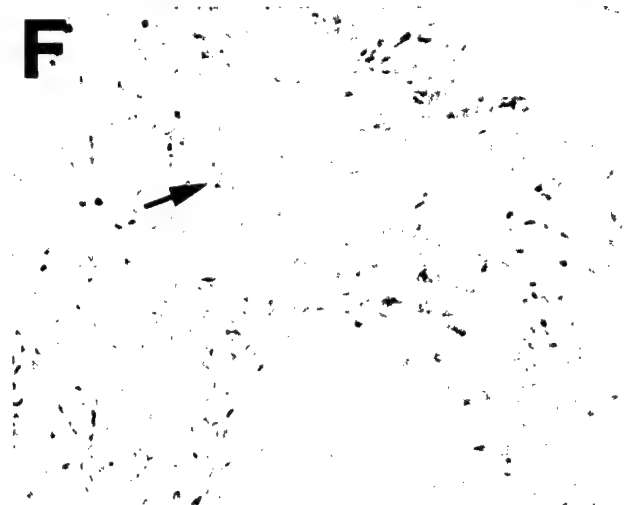
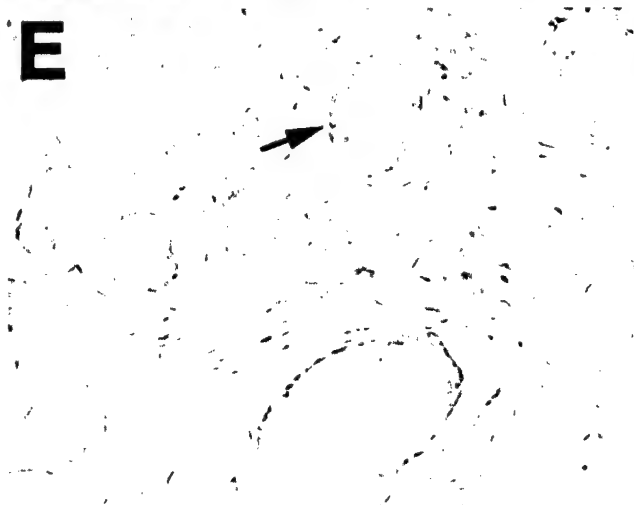
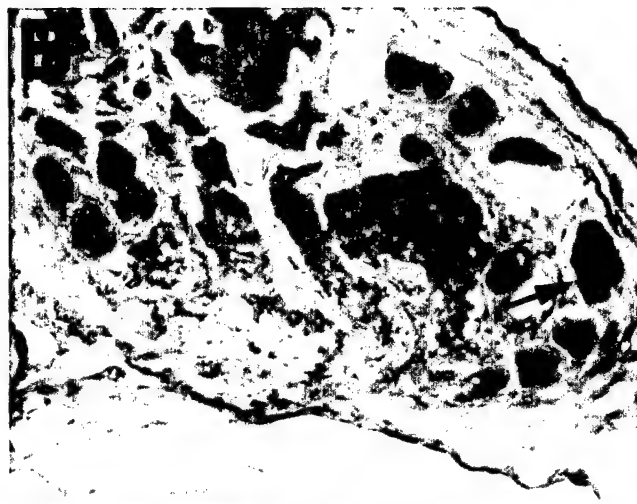
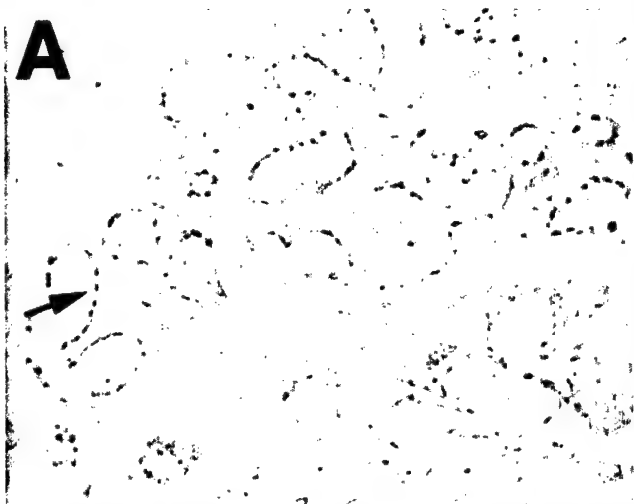


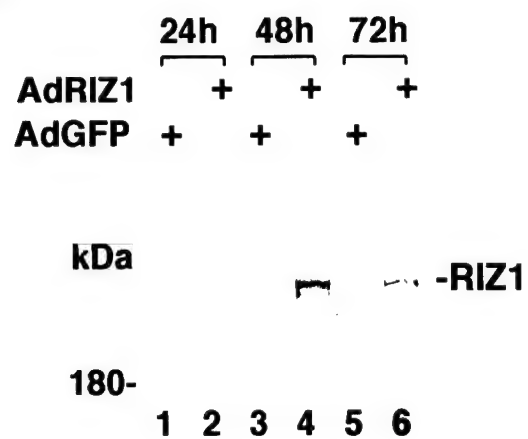
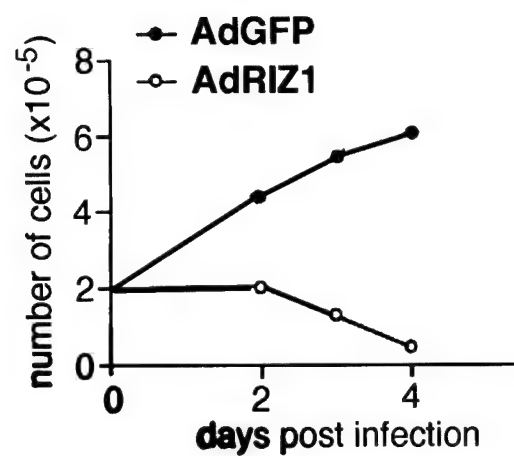
B



C



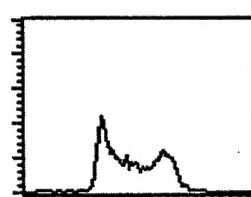
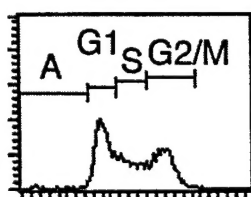


A**B**

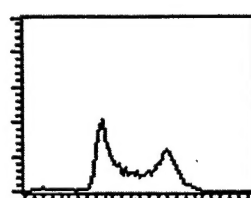
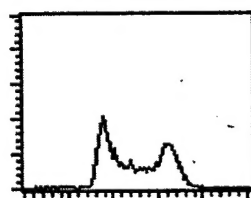
C

AdGFP
U2OS

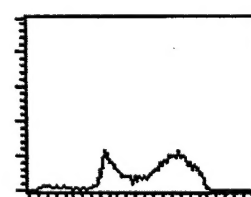
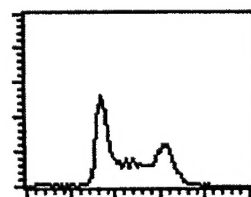
AdRIZ1
U2OS



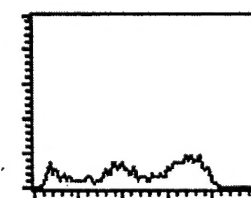
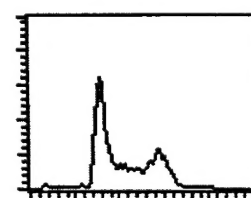
0h



24h

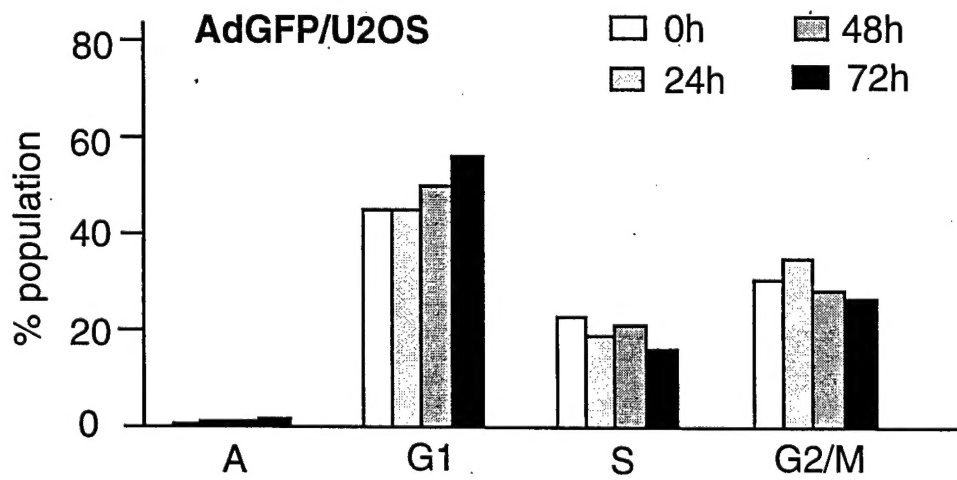
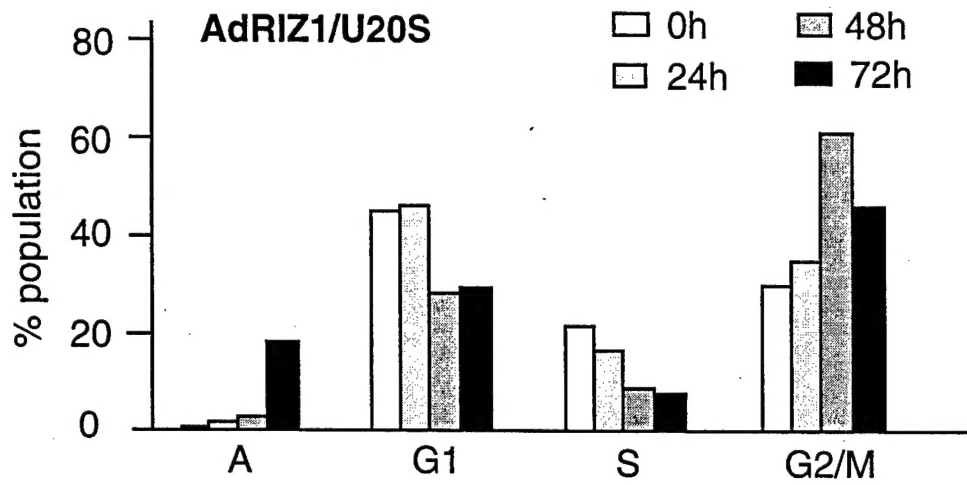


48h



72h

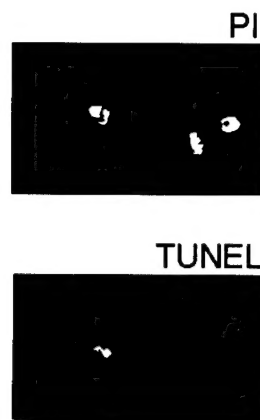
D

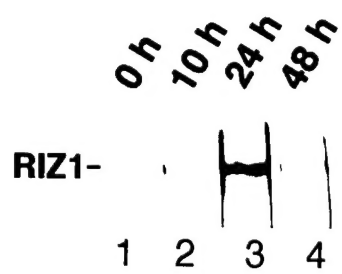


E



F



A**B**